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Evolutionary conserved role of *ptf1a* in the specification of exocrine pancreatic fates

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Abstract

We have characterized and mapped the zebrafish *ptf1a* gene, analyzed its embryonic expression, and studied its role in pancreas development. In situ hybridization experiments show that from the 12-somite stage to 48 hpf, *ptf1a* is dynamically expressed in the spinal cord, hindbrain, cerebellum, retina, and pancreas of zebrafish embryos. Within the endoderm, *ptf1a* is initially expressed at 32 hpf in the ventral portion of the *pdx1* expression domain; *ptf1a* is expressed in a subset of cells located on the left side of the embryo posteriorly to the liver primordium and anteriorly to the endocrine islet that arises from the posterodorsal pancreatic anlage. Then the *ptf1a* expression domain buds giving rise to the anteroventral pancreatic anlage that grows posteriorly to eventually engulf the endocrine islet. By 72 hpf, *ptf1a* continues to be expressed in the exocrine compartment derived from the anteroventral anlage. Morpholino-induced *ptf1a* loss of function suppresses the expression of the exocrine markers, while the endocrine markers in the islet are unaffected. In *mind bomb* (*mib*) mutants, in which *delta*-mediated *notch* signalling is defective [Dev. Cell 4 (2003) 67], *ptf1a* is normally expressed. In addition, the *slow-muscle-omitted* (*smu*) mutants that lack expression of endocrine markers because of a defective *hedgehog* signalling [Curr. Biol. 11(2001) 1358] exhibit normal levels of *ptf1a*. This indicates that *hedgehog* signaling plays a different genetic role in the specification of the anteroventral (mostly exocrine) and posterodorsal (endocrine) pancreatic anlagen.

Keywords: Zebrafish; Pancreas; Exocrine; Endocrine; Ptf1a; Trypsin; Insulin; Hedgehog; Delta; Notch

Introduction

The vertebrate pancreas is an endodermal-derived organ comprised of two main components: the endocrine compartment that secretes hormones in the bloodstream and controls glucose homeostasis, and the exocrine compartment that secretes the main digestive enzymes in the gut lumen. The endocrine tissue is composed of four different cell types: alpha-, beta-, delta-, and PP-cells secreting glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. The exocrine tissue is composed of acini, specialized in enzymes production, and ducts secreting bicarbonated water and vehiculating the pancreatic enzymes to the intestine. As the lack of insulin causes diabetes (a widespread human disease), and pancreatic cancer is one of the most invasive and fatal carcinomas, pancreas has become a model for cellular and tissue differentiation studies and recently much attention has been focused on the genetic program required for the differentiation of each cell type (Edlund, 2002; Kim and MacDonald, 2002).

One of the early decisions in pancreatic cell differentiation is between endocrine and exocrine fates, and *ptf1a* is one of the genes essential for exocrine differentiation. Ptf1a was first detected in nuclei of pancreatic exocrine-type cultured cells during an extensive footprinting analysis of the promoters of digestive enzymes (Cockell et al., 1989), and its isolation led to the characterization of a gene encoding a bHLH transcription factor of 48 kDa (Krapp et al., 1996). Gene-targeting experiments showed that acinar development does not occur in *ptf1a* −/− mouse embryos

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(Krapp et al., 1998). Moreover, conditional targeting and lineage tracing experiment in mouse have evidenced a role of *ptf1a* in converting intestinal to pancreatic progenitor cells (Kawaguchi et al., 2002). The value of zebrafish in the dissection of genetic requirements for endoderm specification, patterning, and differentiation has been amply demonstrated in recent years (Bally-Cuif et al., 2000; Biemar et al., 2001; Dickmeis et al., 2001; Kikuchi et al., 2001; Peyrieras et al., 1998; Stainier, 2002; Tiso et al., 2002). During zebrafish development, the first endocrine pancreatic cells arise at 15 h postfertilization (hpf) from the dorsal aspect of the developing gut (Argenton et al., 1999; Biemar et al., 2001). Recently, using a transgenic zebrafish line that express GFP throughout the endoderm, Field et al. (2003) observed that a second pancreatic bud appears later, at 34 hpf, giving rise to the exocrine tissue and to sparse "late" endocrine cells as well. Then, the two pancreatic buds merge to form the pancreas (Field et al., 2003). However, no molecular markers of the anteroventral anlage have been so far identified.

In this study, we have isolated, characterized, and mapped the zebrafish *ptf1a* gene and analyzed in fine details its expression during early development. We show that *ptf1a* is an early marker of the anteroventral pancreatic bud. In addition, analysis of *mind bomb* (*mib*) and *slow-muscle-omitted* (*smu*) mutant embryos indicate that specification of the two pancreatic anlagen is under different genetic controls. Finally, using the morpholino-antisense technology, we show that *ptf1a* is required in the control of zebrafish exocrine pancreas development and differentiation.

Material and methods

Cloning and radiation hybrid mapping of ptf1a

The *p48-ptf1a* gene was identified by sequence similarity search at the Ensemble Zebrafish Genome Server (Wellcome Trust Sanger Institute/EBI) and at the GenBank database. Protein sequence alignment of Ptf1a was obtained with the ClustalW program (Thompson et al., 2000).

Total RNAs were extracted from the pancreas of 10 adult zebrafish dissected in TRIzol (Gibco/Life Technologies), purified with DNaseI and quantified by agarose-gel electrophoresis. mRNA was then retro-transcribed and amplified with the Access RT-PCR System kit (Promega) using two oligos specific for *ptf1a* (5′-TTGTTGTTACTGGGCAACAC-3′ and 5′-CACAAACATGATTGCCAGTG-3′) yielding a 988-bp cDNA product. To determine the position of *ptf1a* onto the zebrafish genetic map, we used the Goodfellow T51 Zebrafish/Hamster Radiation Hybrid Panel (Invitrogen). PCR screening of the panel was made using the two primers (5′-TTGTTGTTACTGGGCAACAC-3′ and 5′-GTTGGTTTAAGGCGTCACTG-3′), and the following protocol: 25 ng template, 500 nM each primer, 100 μM each dNTP, 2 mM $MgCl_2$, 1 unit Taq DNA Polymerase (Promega) in 20 μl of PCR reaction. Thirty cycles of PCR were completed: 94°C for 1 min, 58°C for 1 min, 72°C for 1 min. Results were submitted to http://zfrhmaps.tch.harvard.edu/ZonRHmapper/instantMapping.htm and processed with the software "RH Instant Mapper". Analyses of synteny were performed using the following databases: WashU (WU) Integrated Maps at http://fisher.wustl.edu/fish_lab/cgi-bin/human_int_map.cgi and NCBI Human/Mouse Homology Maps and LocusLink at http://www.ncbi.nlm.nih.gov/.

Fish maintenance and microinjections

Embryos were grown and staged at 28.5°C according to standard rules and procedures (http://ZFIN.org). The *mib*^{ta52b} (Itoh et al., 2003) *smu*^{b641} (Varga et al., 2001) mutant strains were kindly provided by Ajay Chitnis and Zoltan Varga, respectively. The GUT::GFP transgenic line (Field et al., 2003) was kindly provided by Didier Stainier. Morpholino anti-sense oligos were obtained from Gene Tools. The sequences of morpholinos used in our experiments were as follows: *ptf1a*-MOa, 5′-AGTGTCCATTTTTGTGCTGTGTTG-3′; *ptf1a*-MOb, 5′-GTAACAACAATCGCCTACTCTTCGA-3′.

The stock solution was diluted to working concentrations of 0.5–3 mg/ml in Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6), before the injection into the yolk of one-cell stage embryos. In all microinjection experiments, native GFP and phenol red were used to check the efficiency of the procedure.

Immunohistochemistry and histology

Whole-mount in situ hybridization

Embryos were fixed in 4% buffered *p*-formaldehyde and dissolved in phosphate-buffered saline. RNA in situ hybridizations were performed essentially as reported in Thisse et al. (1993). Digoxigenin and fluorescein-labeled (Roche) antisense probes were synthesized for *ptf1a* (this work), *mnr2a* (Wendik et al., 2004), *trypsin* (Biemar et al., 2001), *insulin*, *glucagon*, and *somatostatin* (Argenton et al., 1999). The *ptf1a* antisense probe was derived from a full-length 988 bp cDNA cloned in a pCRII-TOPO plasmid (Invitrogen). The plasmid was cut with *Xho*I enzyme and transcribed by SP6 polymerase (Promega). Control- and morpholino-injected embryos have been hybridized in the same batch as the control embryos had the tip of the tail artificially cut. At least 20 morpholino-injected embryos have been analyzed in each batch and about 90% of them exhibited the phenotype shown in figures.

Image acquisition and elaboration

All the pictures were acquired from microscope phototubes using a Leica DC500 photocamera and were processed with Adobe Photoshop software.

Results

Zebrafish ptf1a isolation and mapping

A sequence highly similar to the mouse and human *PTF1a-p48* genes was identified in the zebrafish genomic Assembly06 at Sanger Institute by homology search. A single copy of *ptf1a* has been found by repeated BLAST analysis of zebrafish and fugu genomic sequence databases. Its exon/intron structure, determined by sequencing, assembling and extensive search in trace repositories (Fig. 1A), turned out to be identical to the murine *Ptf1a* gene. Synthetic oligonucleotides, designed from the deduced mRNA sequence, were used for its amplification from cDNA. Sequencing of a fragment amplified from adult zebrafish pancreatic cDNA using specific oligonucleotides, confirmed that *ptf1a* is expressed in the adult pancreas (not shown). The high level of amino acid identity (186/265 aa)

A

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 cacgcgactatggatgtagttataagagtcctcgcgtgccgactttcaaactgaactaactcttgagcatcaacaggaaaatatcga
agagtatgcgattgttgttactgggcaacaccaacacagcacaaaaaATGGACACTGTGTTGGATCCATTCACAGGCTTAGACTCTTT
CTCCTCCTCTTATTTCGACGACGATGACTTCTTTACGGACCACTCTTCGAGAGACCACTTGGACACAGACGACTTTCTGGACGAGGAT
GTCGATTTTCTTACCAACCAAATTCAAGAATATTACAAGGATAGTCGGATATCACAGGACGGAGATTATTGTGACGTTGGCAACTTCT
CCTTTTCCTCGTCATCTTCGACTTTTTCTTACGGATGCGCGGACAGCACCTCAGAGCTGTCCCCTCACAGAGACGGAGGCTTACTGAA
GAGGCGGAGGCGCATGAGGTCTGAAGTGGAGATGCAACAGTTGCGTCAAGCTGCCAACGTCCGGGAGCGACGGAGGATGCAGTCCATT
AACGATGCTTTCGAGGGACTGCGATCTCACATCCCAACACTGCCATACGAGAAGAGACTTTCAAAAGTTGACACTTTACGCCTAGCAA
TTGGCTACATAAACTTTCTTGCGGAACTGGTGCAGTCGGACATGCCAATTCGGAATCCACACAGTGACGCCTTAAACCAACCGAAAAA
AGTCATCATTTGCCACCGAGGAACAAgtatgtatatcattgcttattattattattattattattattatattattattattatt
attatcttgttattaaaacaactctttccttttttttctttcagGATCCCCATCTCCAAATGACCCAGACTACGGGTTGCCTCCTCTC
GCAGGACACTCTCTTTCATGGACTGATGAGAAGCAGTTAAAAGACCAGAATATCATTCGGACAGCGAAAGTCTGGACACCAGAGGACC
CACGAAAGTTGCACTTGAAATCATCAATAAACAATATTGAGAATGAACCCCCCTTTAATTTCATTTCCTAAggcaatgttgctattag
tatcccgtatatacacattgtgtacaactgaattgtatggatatttccatgttttgtttatttgtaataattatgggactatctgtgt
tcagcctttagtttgaatgaagataaggccactggcaatcatgtttgtgtcgtatttaattgttttaattgtgctgtaatatatattt
tctaatttaatacggattttatctcaaacaaaaactatttttatacaaacggaacacgaaatttgtctatatttatactgatatgatg
atccaataaaaatttttttatttcactcacgtttgtttgaatttttt
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B

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mouse           MDAVLLEHFPGGLDTFPSPYFDEEDFFTDQSSRDPLEDSDELLGDEQAEVEFLSHQLHEY 60
human           MDAVLLEHFPGGLDAFPSSYFDEDDFFTDQSSRDPLEDGDELLADEQAEVEFLSHQLHEY 60
zebrafish       MDTVL-DPFTG-LDSFSSSYFDDDDFFTDHSSRDHL-DTDDFLDED---VDFLTNQIQEY 54
                **:** : *.* **:*.*.***::*****:**** * * *::* ::   *:**::*::**

mouse           CYRDGACLLLQPAPSAAPHALAPPPLGDPGEPEDN--VSYCCDAGAPLAAFPYSPGSPPS 118
human           CYRDGACLLLQPAPPAAPLALAPPSSGGLGEPDDGGGGGYCCETGAPPGGFPYSPGSPPS 120
zebrafish       -YKD-------------------SRISQDGD---------YCDVGN----FSFS--SSSS 79
                 *:*                   .  .  *:          *:.*     *.:*  *..*

mouse           CLAYPCA---AVLSPGARLGGLNGAAAAAAARRRRRVRSEAELQQLRQAANVRERRRMQS 175
human           CLAYPCAG-AAVLSPGARLRGLSG-AAAAAARRRRRVRSEAELQQLRQAANVRERRRMQS 178
zebrafish       TFSYGCADSTSELSP-HRDGGLLK--------RRRRMRSEVEMQQLRQAANVRERRRMQS 130
                 ::* **   : ***  *  **          ****:***.*:***************

mouse           INDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGSGAGGCGGPGG 235
human           INDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLRGGGAGGCGGPGG 238
zebrafish       INDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLAELVQSDMPIRNPHS-------- 182
                ************************************:****:*:*:::.  :

mouse           SRHLGEDSPGNQAQKVIICHRGTRSPSPSDPDYGLPPLAGHSLSWTDEKQLKEQNIIRTA 295
human           GGRLGGDSPGSQAQKVIICHRGTRSPSPSDPDYGLPPLAGHSLSWTDEKQLKEQNIIRTA 298
zebrafish       -------DALNQPKKVIICHRGTRSPSPNDPDYGLPPLAGHSLSWTDEKQLKDQNIIRTA 235
                       .. .*.:***************.**********************:*******

mouse           KVWTPEDPRKLNSKS-FDNIENEPPFEFVS 324
human           KVWTPEDPRKLNSKSSFNNIENEPPFEFVS 328
zebrafish       KVWTPEDPRKLHLKSSINNIENEPPFNFIS 265
                ***********: ** ::*********:*:*
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C

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retention profile for zebrafish ptf1a
00000000000000000000010000001000000000000100000
00000000101000100020001000000001002111000000100
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Fig. 1. Identification and characterization of the *ptf1a* sequence in the zebrafish genome. (A) A genomic clone containing *ptf1a* coding sequence is present in the zebrafish assembly 06 at Sanger Institute. The *ptf1a* coding sequence (from the ATG to the TAA stop codon) is in capital letters, the identified intron is in small caps. Genetic signals (TATA box, exon/intron consensus boundaries, polyadenylation signal) are underlined. The oligonucleotide sequences used for amplification of *ptf1a* cDNA from pancreatic mRNA are also underlined. (B) Alignment of the zebrafish putative *Ptf1a* amino acid sequence with its human and mouse orthologues. The bHLH class II domain is in bold. (C) Retention profile of the *ptf1a* clone in the Goodfellow RH-mapping panel.

between the zebrafish and human proteins confirmed that the putative *ptf1a* is the bona-fide zebrafish *ptf1a* gene (Fig. 1B). The chromosome position of *ptf1a* was calculated by radiation hybrid mapping. On the basis of the retention profile (Fig. 1C), the zebrafish *ptf1a* gene was positioned on chromosome 2, at 5cR from the z13281 marker, and at the position 46 cM on the WUZGR chromosome 2 integrated map (2:46). The known genes closest to the meiotic marker z13281 are *myom1* and *colec12*.

Zebrafish ptf1a is expressed in the CNS and the pancreas

The expression of *ptf1a* was analyzed by in situ hybridization in zebrafish embryos and larvae at various stages of development. As shown in Fig. 2A, *ptf1a* mRNA was first detected at the 12-somite stage in the dorsal part of the neural tube at the level of the hindbrain (Fig. 2A). At 24 hpf, the hybridization signal was still restricted to the dorsal hindbrain, but the *ptf1a* expression domain had expanded extensively (Fig. 2B). At 48 hpf (Figs. 2C, D), *ptf1a* transcripts were detected in the pancreas, slightly on the right side of the body (Fig. 2C), in addition to the central nervous system (CNS). In the latter, the *ptf1a* probe labeled the retina, a transverse cluster of cells in the dorsalmost area at the midbrain–hindbrain boundary (MHB) that corresponds to the primordium of the cerebellum, and two parallel stripes of cells, located dorsally on each side of the hindbrain, that merged anteriorly with the MHB cluster. At 72 hpf (Fig. 2E), *ptf1a* hybridization signals were restricted to the pancreas, in which the *ptf1a* expression domain, in the form of a club with a hole, reproduced exactly the distribution of the exocrine tissue that at this stage surrounds the islet of endocrine cells (see Figs. 3C, 4G, and Biemar et al., 2001).

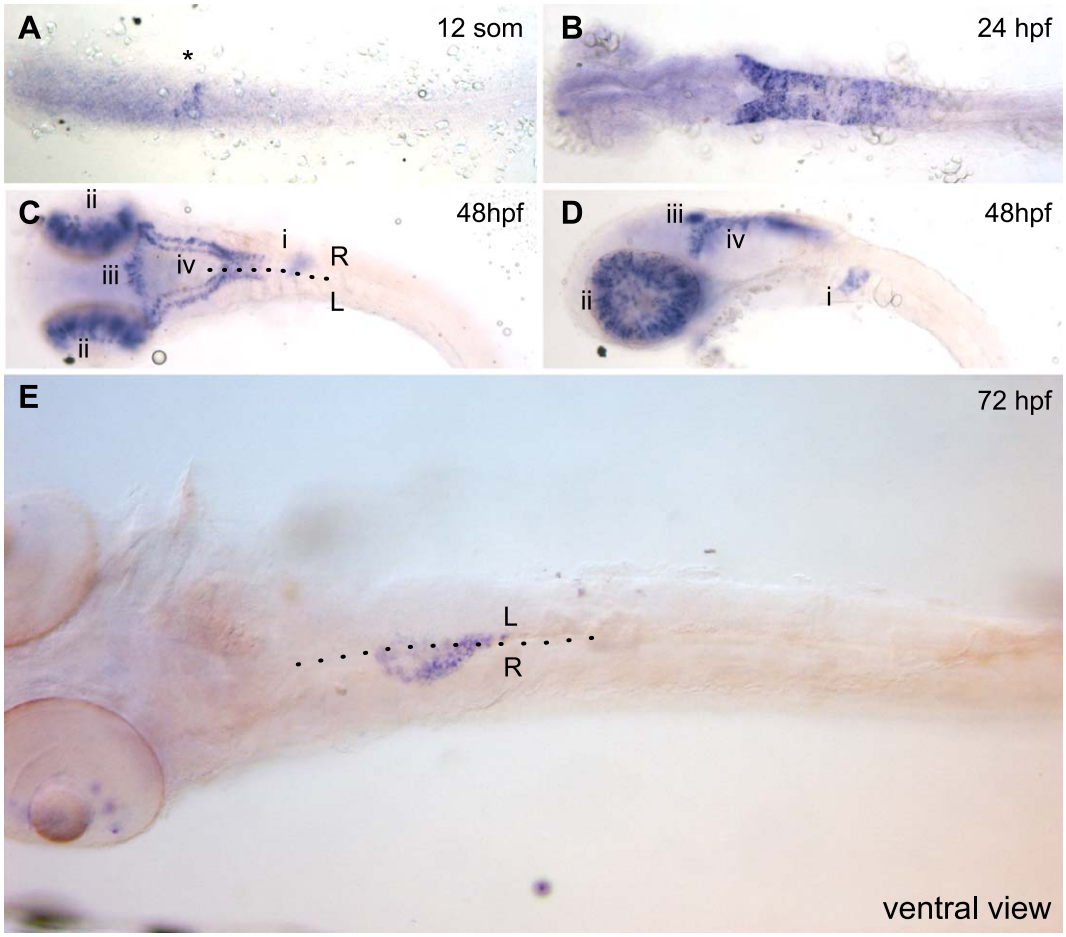

Fig. 2. Whole-mount in situ hybridization with a *ptf1a* antisense probe on zebrafish embryos at different developmental stages. (A and B) *ptf1a* is first detected in the hindbrain of 12-somites zebrafish embryos in the prospective hindbrain area. Some expression is also present in brain regions anterior to the hindbrain. At 24 hpf, the *ptf1a* expression of the hindbrain has expanded to posterior regions. (C and D) At 48 hpf, *ptf1a* is expressed (i) in the pancreas, which has already reached a right side position; (ii) in the retina; (iii) in a transversal line of cells in the dorsalmost midbrain–hindbrain boundary, the prospective cerebellum; (iv) in two parallel lines of cells in the dorsalmost hindbrain. (E) At 72 hpf, *ptf1a* only labels a group of cells positioned in the right side of the embryo at the pancreas level (see also next figure) and arranged as a club with a negative hole in the head. (A–C) are dorsal views, (D) is a lateral view and (E) is a ventral view.

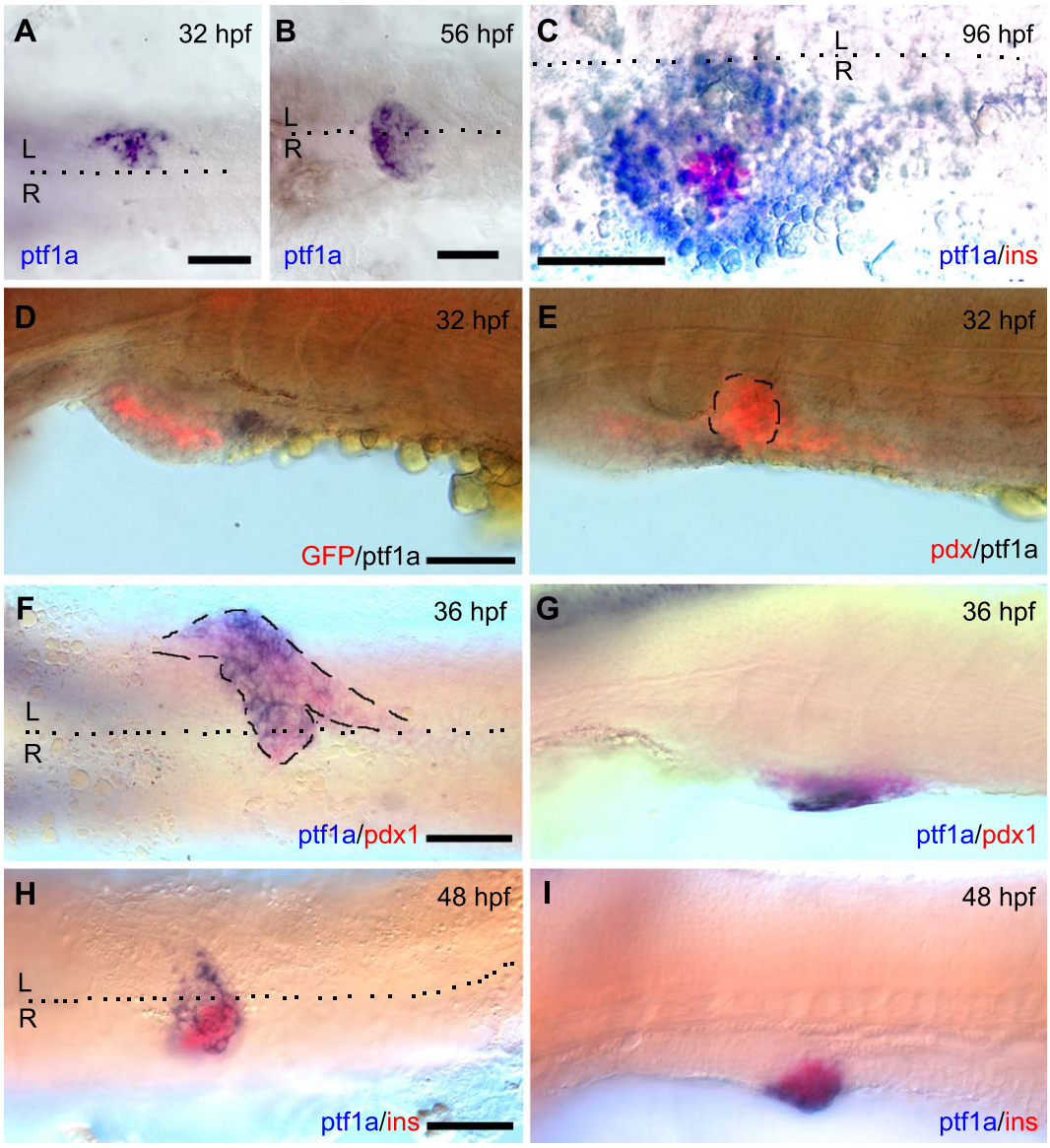

Fig. 3. Expression profile of *ptf1a* in the pancreas region of zebrafish embryos fixed at different developmental stages. (A and B) *ptf1a* expression (in dark) is first detected at 32 hpf in a cluster of cells on the left (L) side of the embryo. At 56 hpf, the expression is detected slightly on the right (R) of the midline in a group of cells engulfing a more posterior negative cluster. (C) At 96 hpf, *ptf1a*-expressing cells (in dark) surround completely the insulin expressing cells of the first endocrine islet (in red). (D and E) At 32 hpf *ptf1a* expressing cells (in dark) are located posteriorly to the liver primordium (D in red) and anteroventral to the *pdx1*-positive "early" islet (E in red, outlined). In order to enhance the *GFP* and *pdx1* expression domains, the pictures represented have been obtained by superimposition of bright field and fluorescent images. (F and G) At 36 hpf *ptf1a*-expressing cells (in dark) are located on the left side of the embryo in a ventral subdomain of the *pdx1* expression domain (in red, outlined in F). (H and I) At 48 hpf, *ptf1a* expressing cells (in dark) have surrounded the insulin-expressing cells of the first endocrine islet (in red). (A–C, F, and H) are ventral views; (D, E, G, and J) are lateral views.

In the pancreas, ptf1a expression is restricted to precursor and differentiated exocrine cells

To determine more precisely the temporal and spatial dynamics of *ptf1a* expression within the endoderm, we performed in situ hybridization of the *ptf1a* probe, alone or in combination with *pdx1* or *insulin* probes, at several time-points between 24 and 96 hpf. As shown in Figs. 3A, F and H, from 32 to 36 hpf *ptf1a* mRNA was detected on the left side of the embryo within the ventral aspect of the *pdx1* expression domain. These first *ptf1a*-expressing cells were located anteriorly to the endocrine islet (Fig. 3E), which is still near the midline at this stage. Subsequently, the *ptf1a* expression domain expanded ventrally towards the right so that it gradually engulfed the endocrine islet, which also moved progressively towards the right side of the embryo (Figs. 3B, F–I). At 96 hpf, the endocrine islet, evidenced by the *insulin* probe, was completely surrounded by *ptf1a*-expressing cells (Fig. 3C).

Using a gut::GFP transgenic line, Field et al. (2003) have recently shown that in zebrafish, the pancreas originates from the fusion of separate posterodorsal and anteroventral anlagen that develop within the *pdx1* expression domain of the gut. The posterodorsal bud, that gives rise to the early endocrine islet, emerges first dorsally from the endoderm on the midline of the embryo and by 24 hpf is already composed of differentiated alpha-, beta-, and delta-cells (Argenton et al., 1999; Biemar et al., 2001). Then, the pancreatic islet relocates progressively on the right side of the embryo. The anteroventral bud, which first gives rise to the exocrine tissue but later to sparse endocrine cells as well, arises at about 34 hpf on the left side from the ventral aspect of the gut just posteriorly to the nascent liver bud (Field et al., 2003). Then, the anteroventral bud grows toward the right side, surrounding progressively the islet, and starts to express the exocrine marker *trypsin* from 72 hpf (see below and Biemar et al., 2001). To determine whether *ptf1a* was expressed in the anteroventral bud, we hybridized 32 hpf gut::GFP transgenic embryos with both *ptf1a* and *GFP* probes. As shown in Fig. 3D, *ptf1a* expression was indeed detected within the *GFP*-positive endodermal domain in cells located just posteriorly to the liver primordium; this region corresponds exactly to the site where the anteroventral pancreatic bud arises (Field et al., 2003).

Our results demonstrate that *ptf1a* is expressed from the very beginning in the cells of the anteroventral bud, and subsequently, in the differentiated exocrine cells as well. By contrast, *ptf1a* is apparently not expressed in the endocrine lineage of the posterodorsal bud. Hence, *ptf1a* represents a marker of both the precursor and differentiated states of pancreatic exocrine cells in zebrafish.

Ptf1a is critical for exocrine pancreas development

The above results suggested that *ptf1a* is involved in the differentiation of the exocrine lineage of the pancreas but does not affect the development of the endocrine cells of the posterodorsal bud. Thus, to get further insights into the functional role played by *ptf1a* in the differentiation of the pancreatic cell types, we examined the effects of its inactivation, achieved by two different antisense morpholinos, on the expression of exocrine and endocrine markers. The two morpholinos, designed on the 5′UTR and the ATG, respectively, produced comparable and synergistic effects.

As shown in Figs. 4A–F, *ptf1a* knock-down affected neither qualitatively nor quantitatively the expression of the endocrine markers *insulin*, *glucagon*, and *somatostatin*. On the contrary, the *trypsin* marker, which is strongly expressed at 72 hpf in the controls (Fig. 4G and Biemar et al., 2001), was not expressed in *ptf1a* morphants (Fig. 4H). This lack of differentiated exocrine cells in *ptf1a* morphants was confirmed by the expression of *mnr2a*, a homeobox gene expressed in the exocrine pancreas and motoneurons of the ventral spinal cord during zebrafish development (Fig. 4I and Meyer et al., in preparation). Indeed, at 72 hpf, no hybridization signal was detected in the pancreatic region of the morphants, although the *mnr2a* expression was unaffected in the CNS (Fig. 4J). Moreover, we have also analyzed *ptf1a* expression in *ptf1a* morphants. As shown in Fig. 5, *ptf1a* expression is normally induced at 36 hpf in the morphants but has

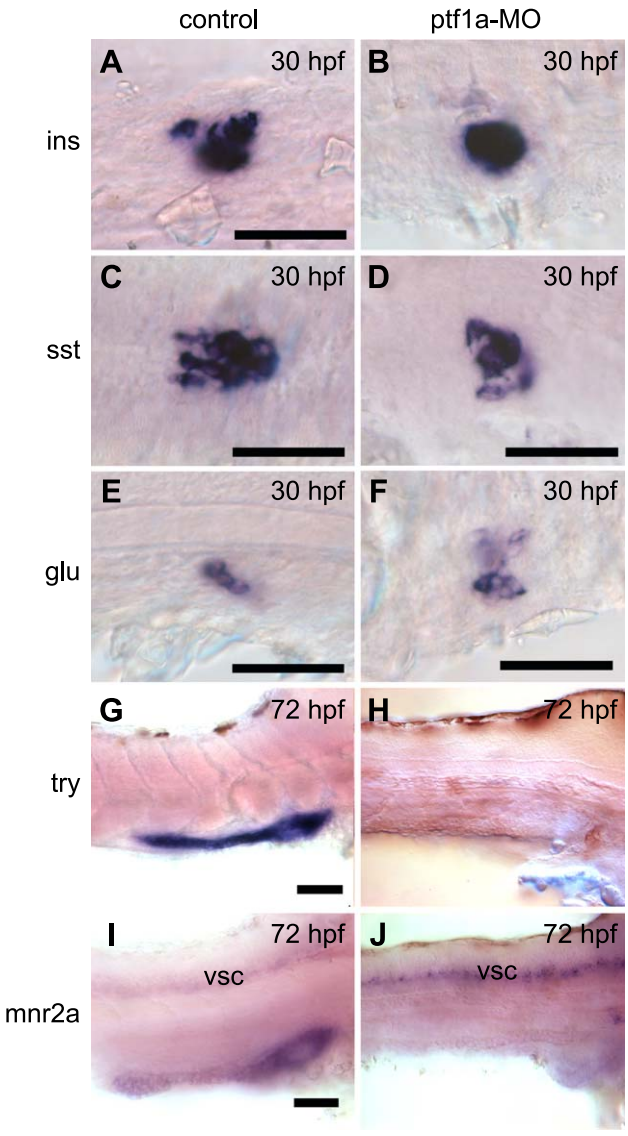

Fig. 4. Whole-mount in situ hybridization with pancreatic markers in zebrafish embryos injected with *ptf1a*-morpholino. Control embryos are on the left side of the panel and *ptf1a*-morpholino-injected embryos are on the right. *ptf1a* functional inactivation does not affect expression of pancreatic endocrine genes (*ins, sst*, and *glu*) while the exocrine markers (*try* and *mnr2a*) are lacking. For the analysis of the expression profile of *ins*, *sst*, and *glu*, embryos were fixed at 30 hpf and presented anterior to the left; for the expression profile of *try* and *mnr2a*, embryos were fixed at 72 hpf and presented in lateral views with dorsal to the top and anterior to the right (the pancreas is on the right side of the embryo). The homeobox gene *mnr2a* is also expressed in motoneurons of the ventral spinal chord. vsc = ventral spinal cord. The scale bar is 50 μm. (A–F) are ventral views; (G–J) are lateral views.

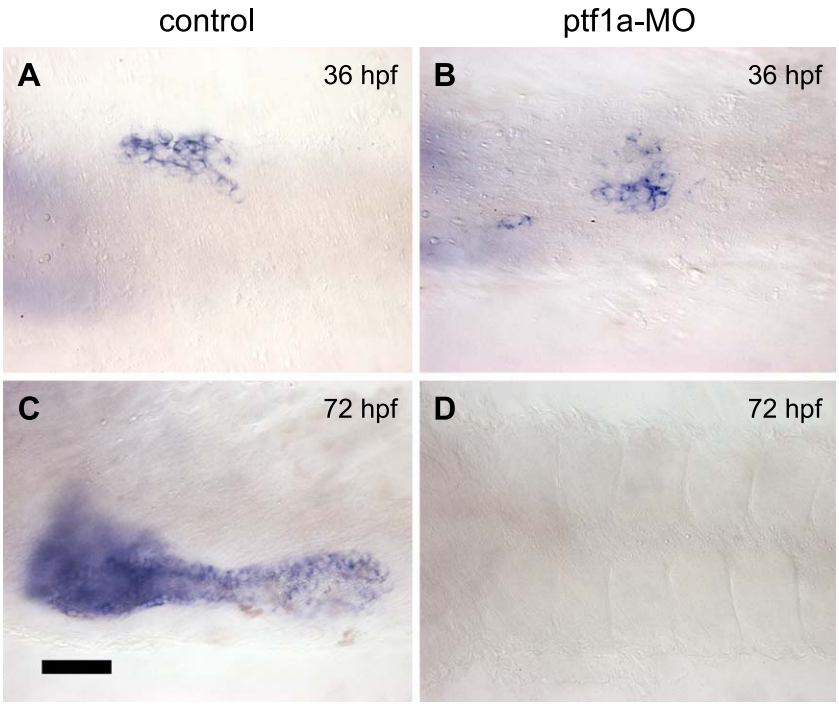

Fig. 5. Whole-mount in situ hybridization with *ptf1a* in zebrafish embryos injected with *ptf1a*-morpholino. *ptf1a* is normally induced at 36 hpf in control (A) as in morphants (B). Conversely, at 72 hpf, *ptf1a* is no longer expressed in morphants (D) compared to controls (C). The scale bar is 50 μm. All embryos are in ventral views with anterior to the left.

disappeared by 72 hpf. This indicates that *ptf1a* is either directly or indirectly involved in the regulation of its own maintenance. These experiments demonstrate that *ptf1a* has no role in the differentiation of the endocrine lineages of the posterodorsal anlage but is critical for the differentiation and maintenance of the exocrine tissue derived from the anteroventral bud.

In mib mutants, ptf1a is expressed and exocrine cells do differentiate

In the mouse pancreas, inactivation of genes involved in *Delta*/*Notch*-mediated lateral specification, such as *Dll-1*, *RBP-Jk*, or *Hes1*, favors the differentiation of endocrine cells at the expense of exocrine cells (Apelqvist et al., 1999; Jensen et al., 2000). In zebrafish *mib* mutants, the genetic inactivation of an ubiquitin-ligase gene results in the incapacity of Delta to activate Notch-mediated lateral specification (Itoh et al., 2003). Hence, to determine whether the Delta/Notch signaling is involved in the specification of the zebrafish exocrine pancreas, we analyzed the expression of *ptf1a* in *mib* mutants. As shown in Fig. 6B, in homozygous *mib* mutants, the expression of *ptf1a* was apparently unaffected, although the morphology of the pancreas was somewhat altered, indicating that in zebrafish, the Delta-mediated Notch signaling does not affect significantly the differentiation of the exocrine pancreas.

Hedgehog signaling is not required for ptf1a expression and for the generation of the anteroventral pancreatic anlage

Two previous studies have shown that in zebrafish, Hedgehog signaling is essential for pancreatic endocrine cell specification (diIorio et al., 2002; Roy et al., 2001). We have therefore investigated the expression of *ptf1a*, *trypsin*, and *insulin* in *smu* mutants, in which a loss of function mutation in the *smoothened* gene abolishes Hedgehog signaling (Varga et al., 2001). In homozygous *smu* embryos, *ptf1a* is expressed within the mid-trunk endoderm in two symmetric patches of cells adjacent to the midline (Figs. 6C, D). This bilateral expression is probably due to the failure of the two lateral stripes of *pdx1*-expressing cells to fuse into a single domain (Roy et al., 2001). Similarly, in 72-hpf *smu* embryos, *trypsin* is also expressed on both sides of the midline (Figs. 6E, F). A previous analysis of mutants with defects of convergence at the midline revealed a similar pancreatic phenotype for *kny* but a normal one for *mil* mutants (Biemar et al., 2001). As previously reported (Roy et al., 2001), we did not detect *insulin*-expressing cells in the majority of 24-hpf *smu* embryos (data not shown), confirming the importance of Hedgehog signaling for the specification of the "early" endocrine cells that arise from the posterodorsal bud. However, in 72-hpf *smu* embryos, *insulin* expression was detected in small clusters of cells located on both sides of the midline (Figs. 6G, H). Taken together, these data indicate that in *smu* mutants, two

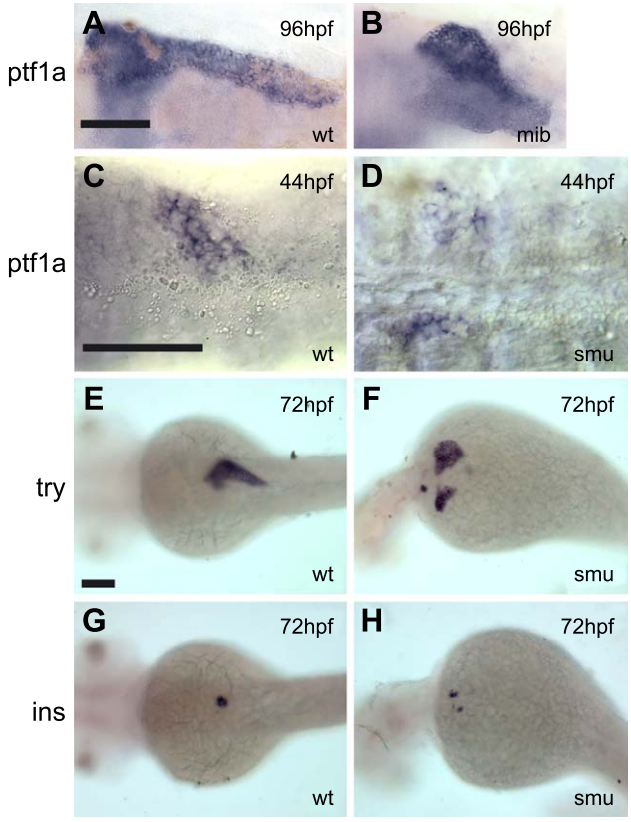

Fig. 6. Whole-mount in situ hybridization with pancreatic markers in *mib* and *smu* mutants and wild-type siblings embryos. Wild-type embryos are on the left side of the panel and mutant embryos are on the right. (A and B) *ptf1a* is expressed at normal level in *mib* mutant embryos (B) at 96 hpf. (C–H) All the pancreatic markers analyzed are expressed in *smu* mutants as two bilateral patches, without any significant decrease of the level of expression, compared to wild-type siblings. Embryos were fixed at 44 hpf (C, D), 72 hpf, and 96 hpf (E–H), and presented in dorsal views with anterior to the left. The scale bar is 100 μm.

anteroventral buds develop, one on each side of the embryo, giving rise to the duplicated exocrine tissue and "late" sparse endocrine cells. Hence, in zebrafish pancreas development, Hedgehog signaling is only required for the differentiation of the posterodorsal bud that generates the "early" endocrine cells.

Discussion

Using a combination of bioinformatic and molecular (RT-PCR) approaches, we have isolated a gene similar to mammalian *ptf1a* from the adult zebrafish pancreas. Its expression pattern, sequence, and exon/intron organization confirmed that this gene is the true orthologue of mammalian *Ptf1a*. Zebrafish *ptf1a* gene maps in the 2:46 region of zebrafish chromosome 2, in a region containing *myom1* and *colec12*, whose human orthologues are located on chromosome 18p11.3. Indeed, according to the GenBank clone AL845362, zebrafish *ptf1a* is physically contiguous to *dlgap1*, also orthologous to a human gene situated on 18p11.3. Thus, *ptf1a* is embedded in a chromosome region that is highly syntenic to the human chromosome region 18p11.3. However, as human *PTF1A* maps on chromosome 10 (in a region highly colinear with mouse chromosome 2), a cytogenetic recombination has involved the *Ptf1a* locus, possibly because of either the teleost genome duplication (Force et al., 1999) or the transposition of a single chromosome region.

The embryonic expression patterns of zebrafish *ptf1a* and its mouse counterpart (Obata et al., 2001) are strikingly similar. In the mouse, at E9.5-10, *Ptf1a* is expressed in the dorsal and ventral pancreas primordia, cerebellum, hindbrain, and spinal cord, in which the expression decreases gradually from the rostral to the caudal end. Similarly, in 48-hpf zebrafish embryos, *ptf1a* is expressed in the pancreas, at the MHB, and in the rhombencephalon. In addition, we also detected *ptf1a* expression in the retina. In zebrafish, the pancreatic expression of *ptf1a* is first detected at 32 hpf. Notably, pancreatic *ptf1a* is first expressed on the left side of the embryo in a region slightly anterior and ventral to the endocrine islet, a structure that in zebrafish is fully differentiated by 24 hpf (Argenton et al., 1999; Biemar et al., 2001). Double hybridization experiments with *pdx1*, a gene expressed in cells committed to duodenal or pancreatic fates in mouse (Ahlgren et al., 1996; Guz et al., 1995; Offield et al., 1996) and zebrafish (Argenton et al., 1999; Biemar et al., 2001), show that *ptf1a*-expressing cells arise from the ventral gut epithelium of the duodenal region. At later developmental stages, *ptf1a* expression extends progressively to the right side of the embryo. In particular, from 48 hpf onwards, *ptf1a*-expressing cells engulf the endocrine islet, a typical feature of the pancreatic exocrine tissue (Biemar et al., 2001). Recently, Field et al. (2003) have described a morphogenetic model of zebrafish pancreas development based on the combined observation of a gut::GFP transgenic zebrafish and the *heart and soul* mutants. They showed that the pancreas derives from two distinct anlagen (posterodorsal and anteroventral) that fuse subsequently; the posterodorsal bud, which arises earlier, gives rise to the first endocrine islet while the anteroventral bud, which develops later, generates first the exocrine tissue (duct and acinar cells), and then also sparse "late" endocrine cells. Our findings support the Field's model and point to *ptf1a* as a molecular/genetic marker for the anteroventral bud. As shown by Field et al. (2003), the anteroventral bud arises as a ridge in the gut at 34 hpf. Hence, our finding that *ptf1a* is expressed in a cluster of cells in the ventral duodenal epithelium at 32 hpf indicates that *ptf1a* is expressed before the budding of the anteroventral primordium. In addition, Field et al. (2003) set the first contact between the two buds at 44 hpf and their complete fusion at 52 hpf. Instead, our observations show that the posterodorsal bud is contacted by 36 hpf and partially surrounded by *ptf1a*expressing cells at 48 hpf. These morphogenetic data are consistent with the possibil-

ity that in zebrafish, as in mouse (Kawaguchi et al., 2002), *ptf1a* is involved in converting intestinal to pancreatic progenitor cells.

In mouse pancreas, the inactivation of genes involved in *Delta-Notch* signaling accelerates the differentiation of endocrine cells at the expense of progenitor/exocrine fates (Apelqvist et al., 1999; Jensen et al., 2000). Moreover, structural interactions between Ptf1a and RBP-Jk led to the speculation that these two transcription factors may cooperate with Notch intracellular domain (Notch-IC) during acinar cell differentiation (Obata et al., 2001). In particular, it was demonstrated that Ptf1a, RBP-Jk and Notch-IC cooperate in the transcriptional activation of the chymotrypsinogen promoter. In *mib* mutant embryos, which are defective in Delta-mediated release of Notch-IC (Itoh et al., 2003), *ptf1a* is expressed in the pancreas and pancreatic cells are normally committed to their exocrine fate. The possibility that the differentiation of exocrine cells in homozygous mutant embryos can be due to maternal *mib* (Itoh et al., 2003) is unlikely. In fact, the early neurogenesis phenotypes of *mib* exon-skipped (zygotic) morphants are very similar to that of *mib* genetic mutants showing that *mib* maternal contribution is not significant at 10 hpf (Itoh et al., 2003), while *ptf1a* is expressed at 32 hpf. It thus appears that ligands of the Delta-class are not implicated in the specification of zebrafish exocrine pancreas. However, we found that DAPT, a gamma-secretase inhibitor that blocks the release of Notch-IC (Geling et al., 2002), impairs exocrine differentiation demonstrating that Notch is involved in the specification of exocrine fates (Zecchin et al. ms. in preparation).

In all vertebrates so far examined, the adult pancreas derives from the fusion of dorsal and ventral buds originating from the foregut. In mammals, the dorsal and ventral pancreatic buds generate independently endocrine and exocrine tissues and are under different genetic control (Kim and MacDonald, 2002). Conversely, in Xenopus and zebrafish, the dorsal bud originates only endocrine cells (Field et al., 2003; Kelly and Melton, 2000). Notably, in the mouse, *ptf1a* is expressed in both the dorsal and ventral buds and is essential for the formation of the exocrine tissue as demonstrated by the absence of such tissue in *Ptf1a* null mutant mice (Krapp et al., 1998). Accordingly, in zebrafish, *ptf1a* is expressed only in the bud that originates the exocrine tissue and its inactivation suppresses this tissue. In addition, *Ptf1a* knock-out in mice affects the spatial organization but not the occurrence of endocrine cells (Krapp et al., 1998), and similarly, our results show that *ptf1a* knock-down in zebrafish does not affect the endocrine lineages of the posterodorsal bud.

In agreement with these data, the promoter of the *trypsin* gene, used as an exocrine marker in our experiments, bears two sequences corresponding to the consensus of Ptf1a binding sites occurring in the promoters of mammalian genes encoding pancreatic enzymes (Cockell et al., 1989; Obata et al., 2001) (Fig. 7). Remarkably, we have also found Ptf1a consensus motifs in the *ptf1a* promoter region (Fig. 7), suggesting that *ptf1a* directly autoregulates its own maintenance (see Fig. 6). Conversely, such motifs do not occur in the 5 kb upstream regions of the *insulin*, *somatostatin*, and *glucagon* genes.

In the mouse, pioneering experiments on pancreatic development have identified a group of "early" endocrine cells that express hormones in the developing buds (Kim and MacDonald). These "early" hormone-producing cells, however, do not express other endocrine markers (such as *Ipf1*, *Nkx6.1*, and *Glut2*), and inactivation of *Ptf1a* or *Pdx1* genes, essential for islet and acinar formation, does not affect their differentiation (Ahlgren et al., 1996; Kawaguchi et al., 2002). As in zebrafish, *pdx1* is expressed in the posterodorsal anlage and its functional inactivation affects heavily the formation of the islet (Yee et al., 2001), it is unlikely that this anlage corresponds to the "early" endocrine cells of the mammalian pancreas. Possibly, from an evolutionary perspective, amniotes have superimposed a *Ptf1a*-mediated genetic program upon a primitive only-endocrine dorsal genetic cascade. Alternatively, common progenitors of amphibians and teleosts may have lost the ability to express *ptf1a* and develop exocrine tissue in the dorsal anlage.

Several studies in mouse and chicken have shown that the dorsal and ventral pancreatic buds are under different genetic controls (Ahlgren et al., 1997; Li et al., 1999) and receive different signals from distinct neighboring tissues (i.e., notochord and lateral plate mesoderm) (Hebrok, 2003; Kumar et al., 2003). Similarly, in zebrafish, formation of the posterodorsal pancreatic bud requires Sonic Hedgehog,

```
                C                   G
Mammalian consensus for PTF    CA CTG..........TTTCCCA
                                 G                   T

Zebrafish trypsin promoter elements:  I  CtCCTGatttatactcTTTCCCTA (-791)
                                     II  CAGCTGa ttat    TTTCCCAT (-1338)
                                         * ****          ******

Zebrafish Ptf1a promoter elements:    I  CAAGTG  gtgtg   ATTCCCAC (-3067)
                                     II  CATGTG  ttgct   ATTCCCAT (-3604)
                                         ** ***          ******

                    Ptf1a consensus:      CANSTG   (N)5-11 WTTCCCAN
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Fig. 7. The zebrafish *trypsin* and *ptf1a* genes that have been analyzed in this study have two putative *Ptf1a* consensus sites in their promoters.

produced mainly by the notochord (diIorio et al., 2002; Roy et al., 2001), while the anteroventral bud is independent of Hedgehog signaling. There is however a significant difference with amniotes in which Hedgehog signaling is a negative regulator of pancreatic cell differentiation (Apelqvist et al., 1997; Hebrok, 2003). Thus, while the function of many pancreatic transcription factors, like *ptf1a* or *pdx1*, has been maintained throughout vertebrate evolution, signaling molecules, such as Hedgehog, play different roles in fish and amniotes.

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